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IMMUNE COMPLEX LEVELS IN CHILDREN WITH SEVERE *PLASMODIUM FALCIPARUM* MALARIA

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Abstract. Malaria infection leads to the formation of circulating immune complexes. However, it is unclear whether these complexes play a role in the pathogenesis of complicated *Plasmodium falciparum* malaria. This study aimed at determining if there are differences in the levels of immune complexes between children with severe malaria-associated anemia and cerebral malaria and between each of these two groups and their respective uncomplicated symptomatic malaria or healthy asymptomatic controls. Children with severe malaria-associated anemia and cerebral malaria had significantly higher immune complex levels than their respective controls, but there were no significant differences in the levels between the two severe malaria groups. In addition, there was an inverse relationship between the hemoglobin levels and immune complex levels in the severe anemia controls, suggesting that immune complexes may contribute to erythrocyte destruction in these children. These results suggest that immune complex levels alone cannot account for the differences in the distinct clinical presentation between severe malaria-associated anemia and cerebral malaria.

INTRODUCTION

Malaria is responsible for more than 1 million deaths per year. Most of these deaths are the result of infection with *Plasmodium falciparum* in sub-Saharan Africa.[1] Nonimmune individuals and children bear most of the morbidity and mortality from malaria, which come as the result of complications such as severe malaria-associated anemia (SMA) and cerebral malaria (CM).[2] The exact mechanisms underlying the pathogenesis of these complications are not fully understood. However, mounting evidence suggests that at least some of these complications may be the result of the normal or aberrant host immune responses against the parasite and not the direct effect of the parasite.[3–5]

Antibody formation is a prominent part of the host immune response against malaria.[5] Parasite proliferation is associated with the generation of soluble plasmodial antigens.[6–8] These antigens may be available for binding by reactive antibodies to form immune complexes (ICs).[9] ICs can activate the complement cascade and stimulate macrophages to produce proinflammatory cytokines,[10,11] both of which can contribute to the pathogenesis of severe malaria.[3,12] Accordingly, many studies have shown that circulating ICs are common during malaria infection.[13–22] However, despite the potential role of ICs in the pathogenesis of severe malaria, there is a paucity of human studies directly addressing this issue and, excluding the work from our own laboratory,[23] we have found only three such studies in the past 25 years.[13,16,19] Two of these studies were limited to adults with cerebral malaria and showed that elevated levels of ICs are present in these patients.[13,19] On the other hand, an earlier study of children with malaria and nonsevere anemia failed to demonstrate the presence of ICs at the time of presentation and instead reported a paradoxical rise in IC levels 1 month after treatment.[16] Our own studies, however, have shown that IC levels are elevated in children with SMA at the time of the diagnosis.[23] In the current study we wanted to determine whether elevated IC levels are present in both children with severe malaria-associated anemia (SMA) and cerebral malaria (CM) in western Kenya and, if true, whether there are quantitative differences between these two groups that could explain their distinct clinical presentations.

MATERIALS AND METHODS

Study design and patient population. This study was reviewed and approved by the Kenya National Ethical Review Committee and by the Human Subjects Research Review Board of the Office of the Surgeon General, U.S. Army. The recruitment of human subjects and study procedures were in accordance with all applicable regulations, and informed consent was obtained from all parents or guardians.

We executed two case-control studies. The demographics of the SMA cases and their uncomplicated malaria controls were recently reported.[23] SMA cases were defined as children with asexual *P. falciparum* parasitemia by Giemsa-stained thick or thin blood smear and hemoglobin $\leq$ 5 g/dL and were recruited from the Pediatric Ward of the Nyanza Provincial General Hospital (NPGH), Kisumu. The NPGH catchment area is the malaria holoendemic region of the Lake Victoria basin, western Kenya. CM cases, defined as children with asexual *P. falciparum* parasitemia by Giemsa-stained blood smear and a Blantyre coma score of $\leq$ 2,[24] were recruited from the pediatric ward of NPGH and the Kisii District Hospital (KDH). KDH is located in the highlands of western Kenya and has seasonal malaria transmission. Consequently, more CM cases were seen at KDH than at NPGH. Because this study also included a separate component to study erythrocyte surface antigens,[23] children were excluded if they had a history of blood transfusion within 3 months preceding enrollment. In addition, cases were excluded if there was clinical evidence of other concomitant infections or malignancy. Two types of controls were recruited and matched by age ± 2 months and gender to each case. Symptomatic controls were children with uncomplicated *P. falciparum* malaria that were recruited from the outpatient clinic of the hospital where the respective case was enrolled. Inclusion criteria for these controls were a Giemsa-stained blood smear positive for asexual *P. falciparum* and an axillary temperature $\geq$ 37.5°C or, in the absence of the latter, two of the following signs or symptoms: nausea/vomiting, irritability, poor feeding, myalgias, or headache. The majority (> 80%) of the children in the symptomatic control groups qualified on the basis of an axillary temperature > 37.5°C. Asymptomatic controls were children who were afebrile and had no signs or symptoms of malaria re-

gardless of the results of the Giemsa-stained blood smear. Asymptomatic controls were recruited from the same village as their matching case. Exclusion criteria for controls were the same as for cases with the addition of any evidence of malaria complication manifested by respiratory distress, palmar or conjuctival pallor, hypotension, seizures, hemoglobin $\leq$ 5 g/dL, or coma. The convalescent time point for SMA cases and their controls was chosen to be 4 months after enrollment to avoid interference of transfused red cells in assays for the determination of red cell surface proteins.[23] The convalescent time point for CM cases and their controls was 1 month after discharge or enrollment.

Collection and processing of blood samples. Approximately 2.5 mL of whole blood was collected at enrollment and at convalescence. Thick and thin blood smears were prepared and stained with Giemsa. The diagnosis of *P. falciparum* parasitemia was confirmed microscopically after scanning a minimum of 200 high power fields. The number of parasites per 500 WBCs was determined. A complete blood count (CBC) was determined using a hematology analyzer (Coulter Corp., Hialeah, FL). The number of parasites/μL was calculated using the white cell density. The plasma was collected, aliquoted into cryovials, and stored at –70°C until use.

Measurement of circulating ICs. To assure comparability, samples reported in a previous study[23] were retested side by side with the additional samples reported in this study. Wells of an Immulon II HB 96-well plate (Thermo Labsystems, Helsinki, Finland) were coated with 10 μg/ml C1q (Sigma-Aldrich, St. Louis, MO) in PBS pH 7.4. After overnight incubation at 4°C, the plates were washed with wash buffer (0.5% Tween 20 in PBS pH 7.4) and blocked for 1 hour at room temperature with blocking buffer (PBS, 0.5% boiled casein, 1% Tween, 0.01% Thimerosal, 20 μg/ml phenol red). Aggregated human IgG (AHG) was prepared by heating 6.5 mg/mL of purified human IgG (Sigma) in PBS at 63°C for 30 minutes followed by fractionation over a Sephacryl S-300 70 × 2.6 cm column (Amersham Pharmacia Biotech, Piscataway, NJ). Fractions from the first peak were pooled and dialyzed overnight against 1 L of PBS. Following determination of total protein concentration, the AHG was aliquoted and stored at –70°C until used. Serial dilutions of AHG were made in PBS for use as standard. Standard and test plasma were diluted 1:60 in dilution buffer (PBS/0.5% boiled casein, 0.5% Tween, 0.01% Thimerosal, 20 μg/ml phenol red) and 100 μL added to duplicate wells followed by incubation for 1 hour at room temperature. The wells were emptied and washed four times with wash buffer. Horseradish peroxidase-conjugated goat anti-human IgG (Kirkegaard & Perry Laboratories, Baltimore, MD) was diluted 1:5000 in wash buffer containing 0.5% boiled casein, and 100 μL was added to each well followed by 1 hour incubation at room temperature. After washing four times, 200 μL of ABTS substrate (Kirkegaard & Perry) was added to each well and incubated for 45 min followed by measurement of the OD_{415nm}. IC level was expressed as micrograms of AHG equivalent per milliliter (μg AHG Eq/mL).

Statistical analysis. Statistical analysis was performed using SPSS for windows version 11.5 (SPSS Inc., Chicago, IL) software package. Analysis of variance (ANOVA) that took into account matching was used to compare the means of all groups within each case-control study at enrollment and at convalescence. Independent samples *t* test was used to compare the means of CM and SMA cases. Parasite densities were log-transformed for the purpose of analysis due to their large variances. Pearson's correlation coefficient was used to examine the relationships between IC levels and parasite density and between hemoglobin levels and IC levels within each group at the time of enrollment. All tests were two-tailed with alpha = 0.05.

RESULTS

Demographic characteristics of the study population. Table 1 summarizes the demographic characteristics of study participants at enrollment. The demographics of SMA cases and their symptomatic controls were reported in a previous study.[23] The mean age (range) for CM cases was 27.5 months (3 months to 8 years) and for SMA cases was 13.6 months (10 months to 7 years). Despite the overlap in ages, the mean difference of 13.9 months, 95% confidence interval 7.1 to 20.7, was significant ($P < 0.01$ by an independent samples *t* test) which is in accordance with previous observations.[25] There were no significant differences in the distribution of ethnic groups between cases and controls.

IC levels were elevated in children with SMA and CM compared with controls. We measured IC levels to identify differences between cases and controls and between children with SMA and CM. Table 2 summarizes the IC levels, the parasite densities, and hemoglobin levels for each group. SMA and CM cases both had significantly higher IC levels

TABLE 1
Demographics of study groups

	Nyanza Provincial General Hospital (NPGH)						Kisii District Hospital (KDH)		
	Severe anemia			Cerebral malaria			Cerebral malaria		
Variable	Cases (N = 58)	SC (N = 57)	AC (N = 58)	Cases (N = 10)	SC (N = 8)	AC (N = 9)	Cases (N = 20)	SC (N = 20)	AC (N = 19)
Mean age (SD) in months	13.6 (14.6)	13.2 (14.2)	13.6 (14.7)	21.0 (8.2)	21.4 (6.8)	22.4 (10.1)	29.4 (18.9)	30.1 (19.1)	31.2 (18.8)
No. females	21	20	21	4	3	4	13	13	12
Nos. by ethnic group									
Luo	49	47	50	10	7	7	0	2	1
Luhya	6	8	6	0	1	2	0	0	0
Abagusii	0	0	1	0	0	0	20	18	17
Others*	3	2	1	0	0	0	0	0	1

SD, standard deviation; SC, symptomatic controls; AC, asymptomatic controls.
* Other ethnic groups include Bukusu, Kalenjin, Kikuyu, Kipsigis.

TABLE 2
Immune complex levels, parasite density, and hemoglobin levels in children with severe malaria and controls

		Severe anemia case-control study (N = 58 cases)				Cerebral malaria case-control study (N = 30 cases)			
	Visit	SMA	SASC	SAAC	SED	CM	CMSC	CMAC	SED
IC (μg AHG Eq/mL)	E	5.1	3.0*	2.5*	0.44	6.3	4.8*	2.7*	0.67
	C	3.0	2.9	2.3	0.63	3.1	2.7	2.0*	0.38
No. parasites $\times 10^{-4}/\mu L$	E	17.6	6.0	1.2*	0.27	15.3	19.3	0.1*	0.29
	C	1.3	1.0	0.7	0.48	1.0	3.2	2.8*	0.50
Hemoglobin (g/dL)	E	4.0	8.6	8.9	NA	8.3	9.2*	10.5*	0.48
	C	9.2	8.9	8.8	0.37	10.5	10.3	10.5	0.34

SMA, severe anemia cases; SASC, symptomatic controls for severe anemia cases; SAAC, asymptomatic controls for severe anemia cases; CM, cerebral malaria cases; CMSC, symptomatic controls for cerebral malaria cases; CMAC, asymptomatic controls for cerebral malaria cases; E, enrollment; C, convalescence; NA, not applicable; SED, standard error of the mean difference between cases and controls.
* $P \leq 0.05$ when compared to means of corresponding SMA and CM cases using analyses of variance taking into account matching between cases and controls.

than their respective controls. The difference between cases and symptomatic controls was more marked in the severe anemia ($P < 0.01$) than in the cerebral malaria ($P \leq 0.05$). Following malaria treatment, the levels of IC in both SMA and CM cases declined, suggesting that they were causally related to the malaria infection. There was no significant difference in the levels of IC between SMA cases and CM cases (independent samples t test, $P = 0.11$).

In both case-control studies, the asymptomatic controls had the lowest parasite density at enrollment because parasitemia was not a factor in the selection of asymptomatic controls, and only a portion of these individuals were parasitemic, 31 (53%) of SMA asymptomatic controls and 11 (39%) of CM asymptomatic controls. There were no significant differences in parasite density between cases and their respective symptomatic controls by the paired t test.

Hemoglobin levels inversely correlate with IC level in SMA controls but not in CM cases or controls. We next explored the relationship between IC levels and parasite density and between hemoglobin levels and IC levels in an attempt to discern some clues as to how IC levels may be contributing to the development of both SMA and CM. Outliers with values four standard deviations above or below the mean were removed from the SMA asymptomatic control group ($N = 1$), the CM cases ($N = 1$), and the CM asymptomatic control group ($N = 1$). Figure 1 presents the scatter plots of IC levels versus parasite density and hemoglobin levels versus IC levels at the time of enrollment in the severe anemia case-control study. There was a positive relationship between IC levels and parasite density in SMA cases (Figure 1b) but not in the other groups. In contrast, there was a negative correlation between hemoglobin levels and IC levels in the asymptomatic control group ($P < 0.001$) (Figure 1e) and a less strong correlation in the symptomatic controls ($P = 0.06$) (Figure 1c). The corresponding results for the cerebral malaria case-control study are summarized in Figure 2, which shows no significant relationship between IC levels and parasite density or between hemoglobin levels and IC levels.

DISCUSSION

ICs can lead to cell and end organ damage by their accumulation on cell surfaces and by initiating the complement cascade resulting in the deposition of complement activation products on cells such as erythrocytes[12] and other organs such as the kidneys.[18,26,27] Also, by cross-linking Fc receptors on macrophages, ICs can stimulate the production of proinflammatory cytokines[10,11] that have been proposed to have a role in the pathogenesis of severe malaria, especially CM.[3] Despite the plethora of deleterious effects that come as a result of IC formation, relatively little research has been done to determine if ICs play a role in the pathogenesis of severe malaria in humans. The main objective of this study was to determine whether children with CM and SMA have elevated IC levels and whether these levels differ quantitatively between these two groups.

We found that IC levels of children with SMA and CM were higher than their respective age and gender-matched controls, but there was no significant difference in the IC levels between the two groups. The IC levels diminished in all groups in response to malaria treatment, suggesting that malaria played an important role in IC formation. In both case-control studies, parasite densities were significantly lower in the asymptomatic groups in part due to the fact that a significant proportion of individuals in these groups were not parasitemic. However, there was no difference in mean parasite densities between cases and symptomatic controls that could account for the differences in IC levels between these groups.

Further evidence that parasite density alone could not account for the increased IC levels in children with severe malaria is the fact that we only found a correlation between parasite density and IC levels in SMA cases. However, we do not feel that the lack of correlation in other groups eliminates parasitemia as a contributing factor to IC formation but rather suggests that, although parasitemia is important in IC formation, other factors may also play a role in determining IC levels. One such factor may be the rate at which opsonized ICs are removed from circulation by erythrocyte complement receptor 1 (CR1, CD35).

CR1 is a complement regulatory protein found on erythrocytes. One of its important functions is to remove opsonized ICs from circulation.[28] We have found that erythrocytes of children with SMA are deficient in this protein at the time of presentation compared with their controls.[23,29] Therefore, the IC removal mechanism of children with SMA may be ineffective at any level of parasitemia and may result in significant IC accumulation in the circulation. Although similar deficiencies have not been observed in erythrocytes of CM cases,[30] it is possible that the IC removal mechanisms in children with CM may be compromised by the binding of CR1on uninfected erythrocytes to infected erythrocytes containing mature parasites (schizonts) to form rosettes,[31,32] leading to ac-

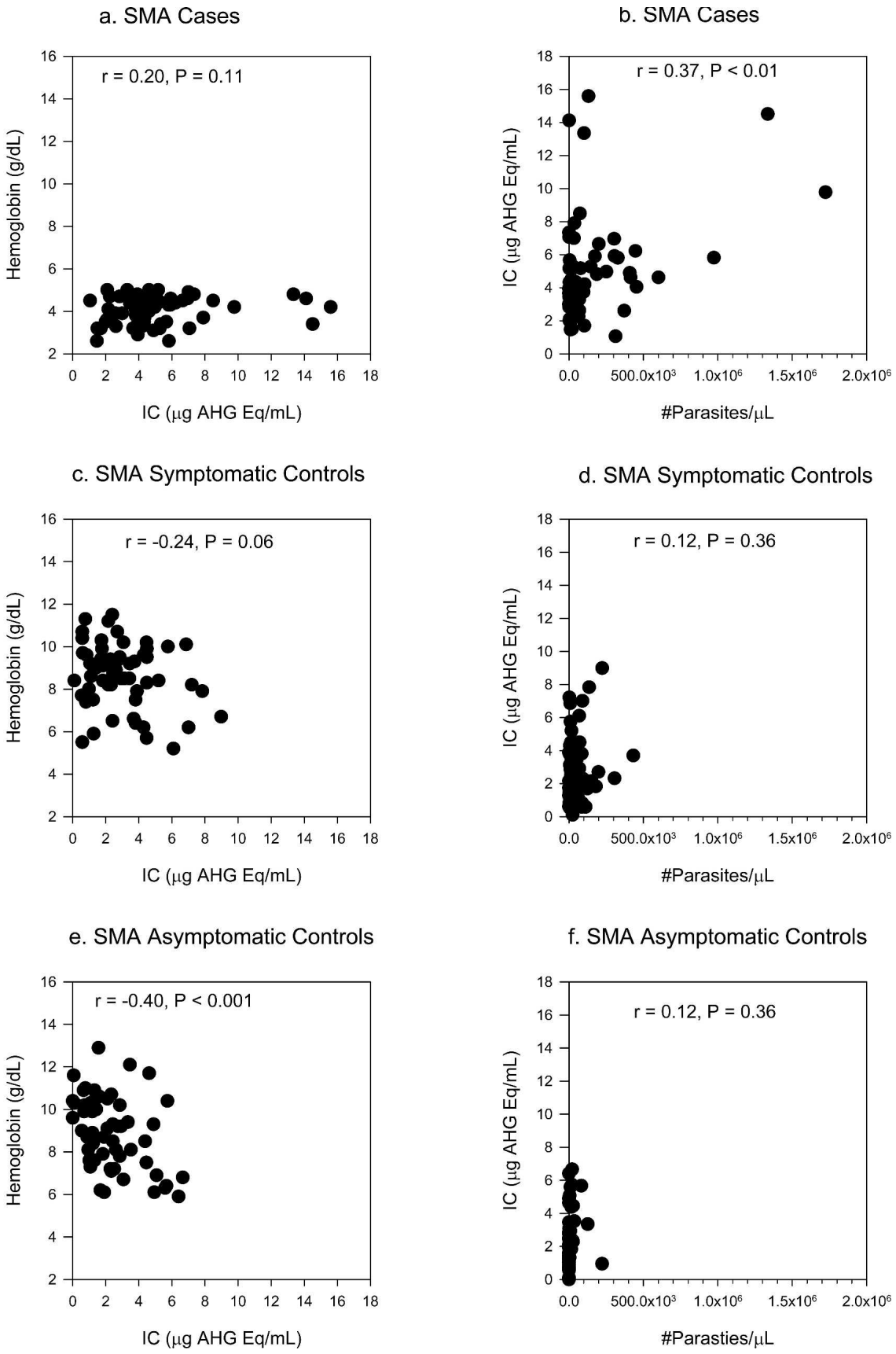

FIGURE 1. Relationships between hemoglobin levels and IC levels and between IC levels and parasite density in children with severe malaria-associated anemia (SMA) and controls at enrollment.

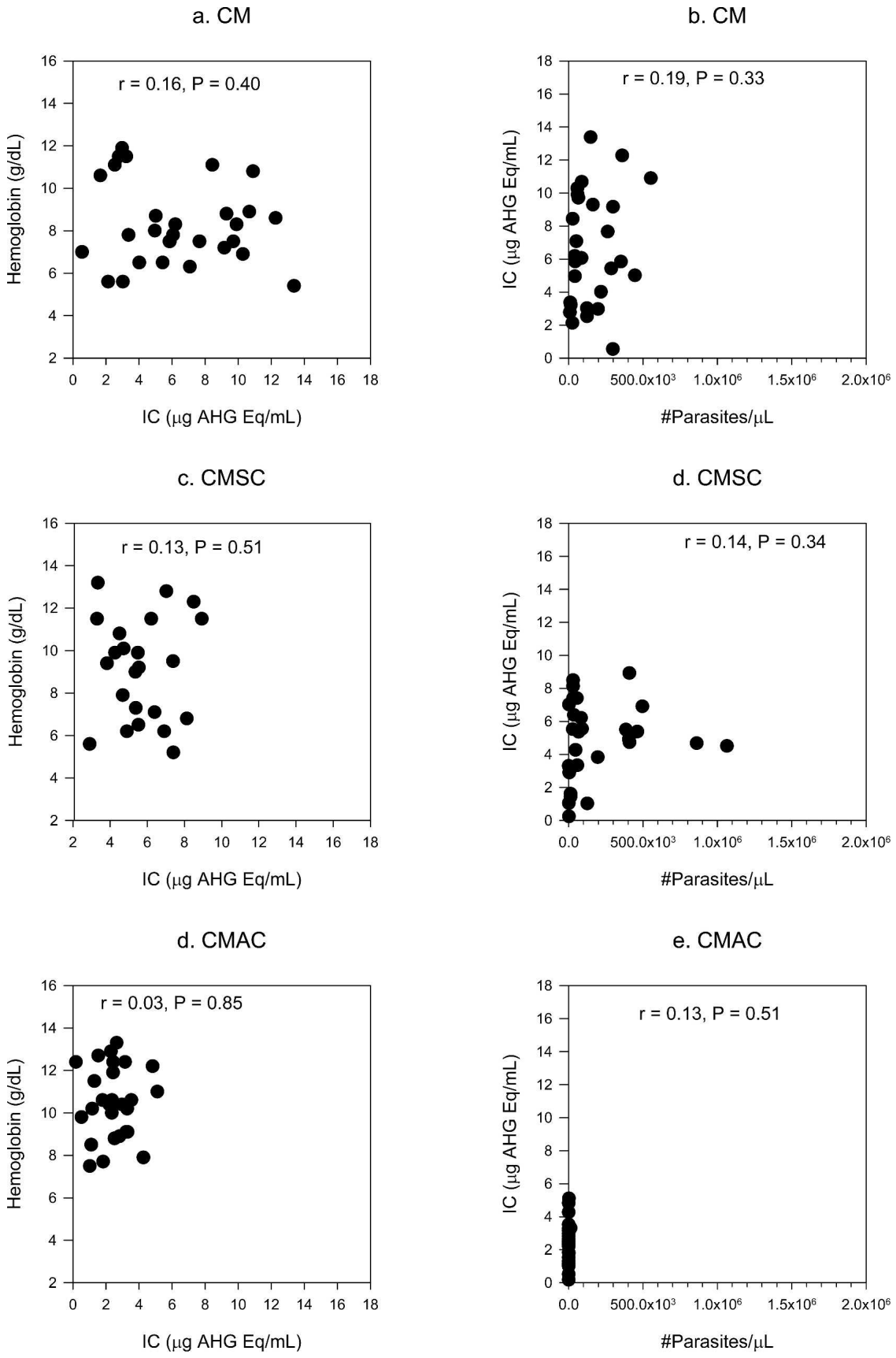

FIGURE 2. Relationships between hemoglobin levels and IC levels and between IC levels and parasite density in children with cerebral malaria (CM) and controls at enrollment. CMSC = symptomatic controls for CM Cases, CMAC = asymptomatic controls for CM Cases.

cumulation of ICs. Studies in our laboratory are currently comparing the erythrocyte IC binding capacity of these groups of children to test these hypotheses.

Of interest is the fact that we observed negative correlations between hemoglobin and IC levels in the SMA symptomatic and asymptomatic controls. This suggests that ICs in these children may play a role in the development of anemia. The lack of correlation between hemoglobin levels and IC levels among the SMA cases may have been due to the extremely low hemoglobin values seen in these children resulting in a very narrow range of hemoglobin levels in which to observe this relationship. ICs can contribute to the development of severe anemia by activating the complement cascade leading to C3b deposition on erythrocytes as bystander effect. Because erythrocytes of children with SMA are deficient in CR1 and CD55,[23,29,30] increased IC levels in these children could translate into increased C3b deposition on their erythrocytes. Although previous studies have shown that no significant hemolysis takes place without CD59 deficiency,[33] increased C3b deposition can lead to increased destruction of erythrocytes by phagocytosis in reticuloendothelial tissues such as the spleen. The crucial role of CR1 and CD55 in preventing C3b deposition on erythrocytes has also been demonstrated in mice with genetically engineered deletions of the homologues of these proteins.[34,35]

Among CM cases and controls we found no relationship between hemoglobin level and IC level, suggesting that erythrocytes of older children such as CM cases and controls may be more resistant to the deleterious effects of circulating ICs. ICs could influence the development of CM in a manner completely different from that proposed for children with SMA. Children with CM may have higher levels of erythrocyte CR1 than other children.[30] This could result in increased rosette formation[31] that could plug cerebral capillaries. In addition, erythrocytes with higher levels of CR1 in CM patients could carry a greater load of IC that could stimulate monocytes in sequestered cerebral capillaries to produce TNF-α and nitric oxide,[36] mediators that have been postulated to have an important role in the development of CM.[37]

A most important finding is that IC levels were elevated in both children with SMA and children with CM. This observation suggests that although ICs may be involved in the pathogenesis of these two conditions, they alone cannot explain their distinct clinical manifestations. The difference between the two groups may lie in the way in which they respond physiologically to increased IC levels. Thus, erythrocytes of children at risk of SMA may have greater susceptibility to IC-mediated complement activation than those of children who are not at risk of this complication. On the other hand, children at risk of CM may have greater susceptibility to IC-mediated stimulation of proinflammatory mediators in the cerebral microvasculature than other children. Alternatively, it is also possible that there may be qualitative rather than quantitative differences in ICs between these groups that could further impact on the clinical presentation. Therefore, it may be worth determining the predominant antibody subclass or isotype in the ICs of these children. Nonetheless, ICs are clearly present in both conditions and are available to cause damage by activating complement and stimulating proinflammatory cytokines. It is tempting to speculate that ICs in these patients may contribute to the systemic pro-inflammatory cytokine picture seen in these conditions, which until now has been singly attributed to the presence of malaria toxin.[38]

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